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TLR4 deficiency does not alter glaucomatous progression in a mouse model of chronic glaucoma

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Glaucoma is a leading cause of irreversible blindness worldwide. Toll-like receptor 4 (TLR4) is a pattern-recognition transmembrane receptor that induces neuroinflammatory processes in response to injury. *Tlr4* is highly expressed in ocular tissues and is known to modulate inflammatory processes in both anterior and posterior segment tissues. TLR4 activation can lead to mitochondrial dysfunction and metabolic deficits in inflammatory disorders. Due to its effects on inflammation and metabolism, TLR4 is a candidate to participate in glaucoma pathogenesis. It has been suggested as a therapeutic target based on studies using acute models, such as experimentally raising IOP to ischemia-inducing levels. Nevertheless, its role in chronic glaucoma needs further evaluation. In the current study, we investigated the role of TLR4 in an inherited mouse model of chronic glaucoma, DBA/2J. To do this, we analyzed the effect of *Tlr4* knockout (*Tlr4*$^{-/-}$) on glaucoma in DBA/2J mice. Our studies found no significant differences in intraocular pressure, iris disease, or glaucomatous progression in *Tlr4*$^{-/-}$ compared to *Tlr4*$^{+/+}$ DBA/2J mice. Our data do not support a role for TLR4 as a treatment target in chronic glaucoma.

Keywords Glaucoma, Toll-like receptor 4 (TLR4), Intraocular pressure (IOP), Neurodegeneration, Neuroinflammation

Glaucoma refers to a complex group of multifactorial diseases characterized by the progressive degeneration of retinal ganglion cells (RGCs) and visual field deficits[1,2]. Elevated intraocular pressure (IOP), genetics, and advanced age are considered the most important risk factors for glaucoma[2,3]. Both neuroinflammation and perturbed metabolism are implicated in the disease. Nevertheless, the mechanisms that lead to neural demise and degeneration in glaucoma are yet to be fully elucidated.

Neuroinflammation is involved in glaucomatous progression[1,2,4–8]. We have previously investigated the processes implicated in the initiation of glaucomatous damage and identified monocyte cell entry into the optic nerve head as an important pathogenic event in DBA/2J glaucoma[4,6,9,10]. Subsequently, macrophages were found to infiltrate axon bundles in the optic nerves of glaucoma patients[11]. Additionally, we and others have shown that mitochondrial dysfunction and impaired metabolism contribute to the development and progression of glaucoma[8,12–23]. In a vicious cycle, pro-inflammatory mediators (released by e.g., infiltrating macrophages) can trigger mitochondrial damage and amplify the neurodegeneration driven by mitochondrial dysfunction[17,24].

Toll-like receptors (TLR) are a family of pattern-recognition transmembrane receptors and a centerpiece of the innate immune response[25]. They can recognize external pathogen-associated molecular patterns (PAMPs) like bacterial lipopolysaccharide (LPS), as well as endogenous damage-associated molecular patterns (DAMPs)[26]. They play a significant role in inflammatory conditions and neurodegenerative diseases[27]. TLR4, in particular, is highly expressed in cells of the central nervous system and the retina, and its activation triggers multiple signaling cascades that lead to the release of inflammatory cytokines and immune modulators[28,29]. TLR4 has been implicated in the regulation of glucolipid metabolism[30]and in mitochondrial dysfunction after LPS-induced TLR4 activation[31]. Additionally, TLR4 activation can induce metabolic changes in macrophages via mitochondrial reprogramming[32].

TLR4 has been studied as a therapeutic target in preclinical models of eye disorders[33–35]. Its activation has been linked to ocular inflammation, retinal damage following acute experimental elevation of IOP to an ischemic level, and retinal/ischemia-reperfusion injury[36–38]. In murine optic nerve crush, an acute model with relevance to glaucoma due to direct axon injury, blocking TLR4 suppressed inflammatory reactions and RGC loss[34,39]. In

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human tissues, increased expression of TLR4 was reported in glaucomatous human trabecular meshwork (TM), a tissue relevant to IOP elevation, and in glaucomatous human optic nerve head[40–42]. Finally, polymorphisms in *TLR4* are suggested to be involved in normal tension glaucoma and primary open-angle glaucoma (POAG), but further data are needed[43–46].

Given the important effects of TLR4 on both metabolism and neuroinflammation, we functionally tested the role of TLR4 in a chronic glaucoma by comparing *Tlr4*-deficient ($Tlr4^{-/-}$) and wild-type ($Tlr4^{+/+}$) littermate mice on a DBA/2J background. DBA/2J mice are a model of hereditary glaucoma and are widely used for glaucoma research. Mutations in two genes impacting melanosomes cause pigment-dispersing iris disease, IOP elevation, and glaucoma in DBA/2J mice[47]. Variants in *PMEL*, a gene with a role in melanosome biology, cause pigmentary glaucoma in humans[48]. Thus, the initiating melanosomal etiology of DBA/2J glaucoma is similar to at least a subset of human pigmentary glaucoma. Importantly, findings in DBA/2J have been extended to POAG, the most common form of glaucoma, with initial promising outcomes in clinical trials[12,15,49,50]. In our experiments, we studied whether TLR4 plays a role in the development of DBA/2J glaucoma. We observed no significant differences in IOP, nerve damage, and other glaucoma-associated phenotypes between $Tlr4^{+/+}$ and $Tlr4^{-/-}$ DBA/2J mice. Our data indicate that TLR4 is not necessary for disease progression in this chronic glaucoma model.

Results

Tlr4 knockout has no effect on pigment-dispersing iris disease or IOP

DBA/2J mice develop a pigment-dispersing iris disease that results in pigment accumulation in the ocular drainage tissues and subsequent IOP elevation. There were no differences in the onset or progression of the iris disease between mice of each *Tlr4* genotype (Fig. 1A). At all ages (analyzed between 4 and 12 months old), both groups followed the usual anterior segment disease progression that we and others have previously characterized for DBA/2J mice[51–55]. DBA/2J mice typically have clear disease by 5 months of age, consisting of transillumination defects that involve progressive depigmentation and iris atrophy[51–53]. At 8 months of age, both $Tlr4^{+/+}$ and

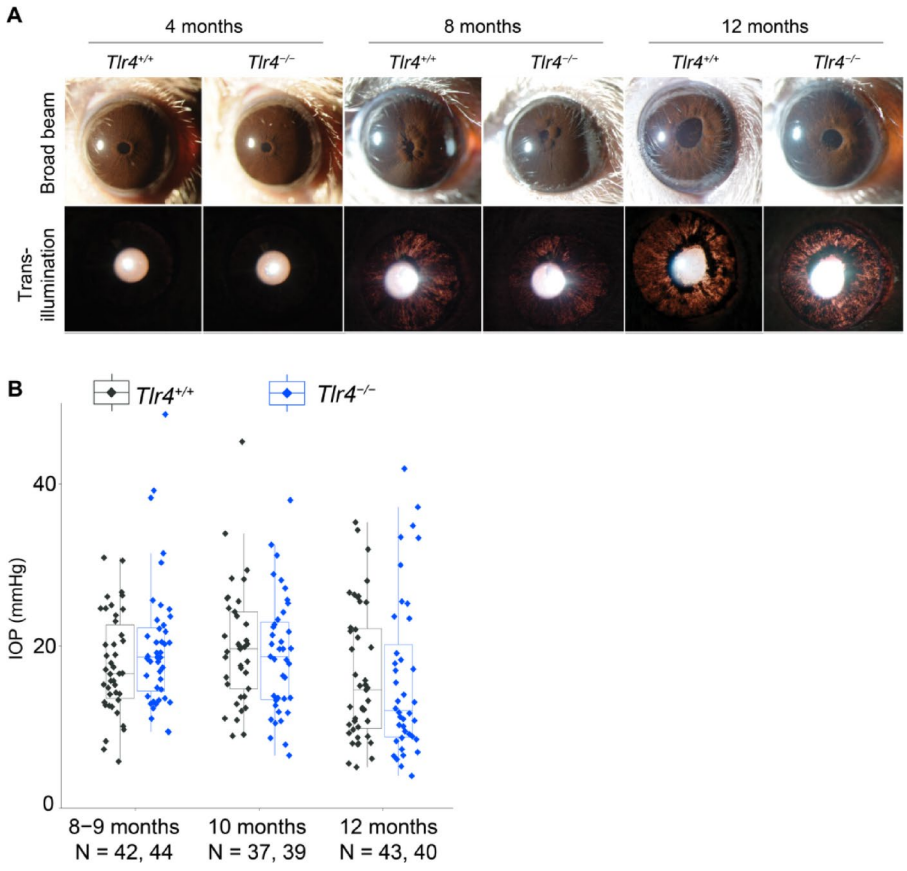

Fig. 1. *Tlr4* deficiency does not influence the iris disease or the IOP phenotype of DBA/2J mice. (**A**) Representative slit-lamp images of $Tlr4^{+/+}$ (wild-type DBA/2J) and $Tlr4^{-/-}$ eyes at 4, 8, and 12 months. The top row shows broad beam illumination, and the bottom row shows transillumination. The glaucoma-related changes in $Tlr4^{-/-}$ mice developed at the same time as in $Tlr4^{+/+}$ mice. At 4 months of age, iris disease is not yet evident in either of the groups. Eight-month-old $Tlr4^{+/+}$ and $Tlr4^{-/-}$ eyes present dispersed pigment and transillumination defects, which become more severe at 12 months of age. $N > 20$ mice per genotype at each age. (**B**) IOP distributions at key ages. There was no significant effect of the genotype on IOP levels at any age (two-way ANOVA; $P = 0.702$).

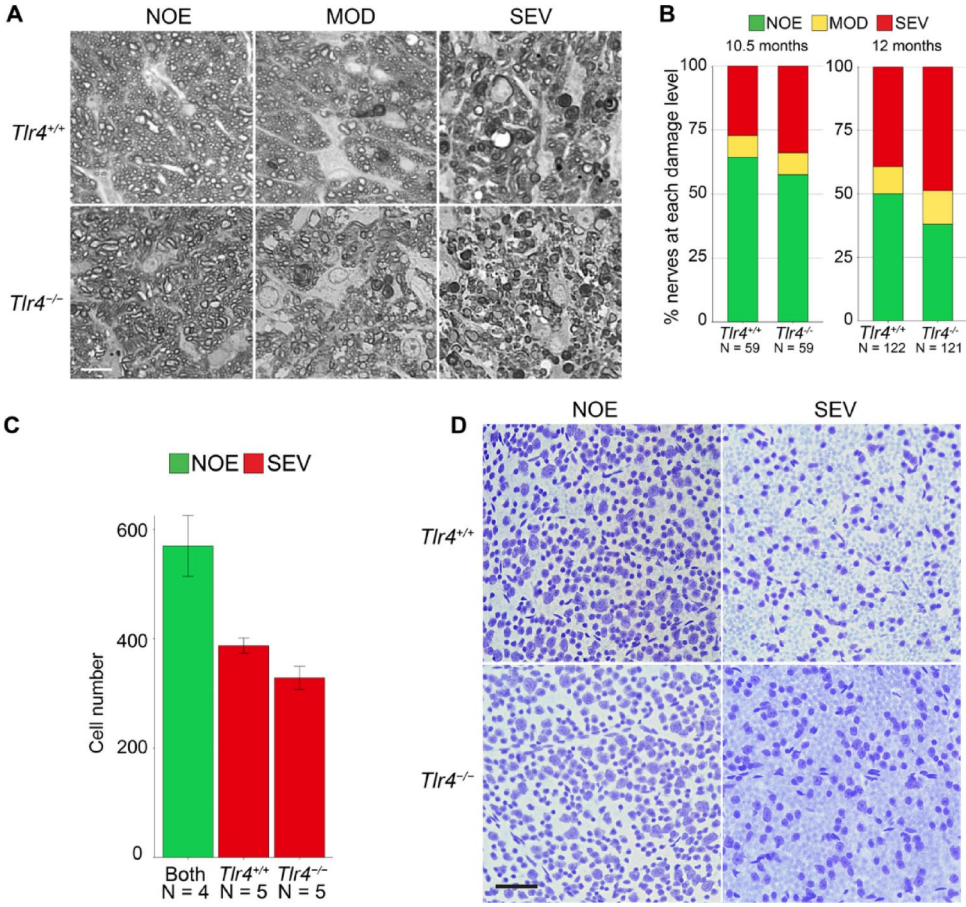

Fig. 2. Lack of *Tlr4* does not affect glaucomatous neurodegeneration. (**A**) Representative images of nerves with no glaucoma (NOE, no/early, see Methods), moderate (MOD), and severe (SEV) damage. Scale bar = 10 µm. (**B**) Frequency distribution of optic nerve damage at 10.5 and 12 months of age. No significant differences were observed between the two genotypes of mice at any age (Fisher's test at 10.5 months, $P = 0.749$; $P = 0.169$ at 12 months of age). (**C**) RGC layer cell counts for eyes with no glaucoma (NOE, both genotypes combined) or severe (SEV, split by genotype) glaucoma based on optic nerve damage. No significant differences in RGC layer cell numbers were observed between eyes of either genotype with severe glaucoma, indicating that somal and axonal damage are not uncoupled by the *Tlr4* mutation (one-way ANOVA and post hoc Tukey's HSD test, $Tlr4^{+/+}$ SEV vs. $Tlr4^{-/-}$ SEV; $P = 0.384$). (**D**) Representative images of the retinas of $Tlr4^{+/+}$ and $Tlr4^{-/-}$ mice with no or severe nerve damage at 40X. Scale bar = 50 µm.

$Tlr4^{-/-}$mice presented peripupillary accumulation of pigment, dispersed pigment, and transillumination defects. The depigmenting iris disease worsened by 12 months of age, with severe transillumination defects and iris atrophy in both groups. In the DBA/2 J model, the IOP is increased in many mice by 8–9 months of age and starts declining after 12–13 months due to atrophy of the ciliary body[52,53]. We did not detect any differences in IOP between $Tlr4^{+/+}$ and $Tlr4^{-/-}$ mice (p-value = 0.702, two-way ANOVA. Figure 1B).

Tlr4 knockout has no effect on RGC numbers or axonal degeneration

To study the role of TLR4 in glaucomatous degeneration, we assessed the effects of *Tlr4* deficiency on optic nerve damage using PPD-stained nerve cross-sections. We evaluated nerves at 10.5 and 12 months of age, two key glaucoma points frequently used for assessing glaucomatous neurodegeneration in this model[4,56] (Fig. 2A-B). There were no significant differences in nerve damage between genotypes at the two ages analyzed. Because the *Tlr4* mutant mice initially had a slightly higher incidence of severe damage at each age, we analyzed an even larger number of 12-month-old eyes. Despite the larger number of nerves, the difference remained statistically insignificant (Fisher's test comparing $Tlr4^{+/+}$ and $Tlr4^{-/-}$ mice at 10.5 months of age, $P = 0.749$; $P = 0.169$ at 12 months of age). At 10.5 months of age, 27% of the $Tlr4^{+/+}$ nerves had severe nerve damage (> 50% axonal loss), and this proportion increased to 39% at 12 months of age. For the $Tlr4^{-/-}$ mice, 33.9% of the nerves had severe damage at 10.5 months of age, which increased to 48.8% by 12 months.

To evaluate potential axonal-somal uncoupling, where RGC somal survival is observed despite axon loss[57–59], we analyzed RGC layer cell numbers in Nissl-stained flat-mounted retinas. To assess uncoupling, we counted and compared retinas of $Tlr4^{+/+}$ and $Tlr4^{-/-}$ eyes that had severe or no glaucomatous damage (Fig. 2C-D). As expected, retinal cell counts in $Tlr4^{+/+}$ and $Tlr4^{-/-}$ mice with severe nerve damage were considerably reduced

with no effect of the genotype on this loss ($P = 0.384$ when comparing *Tlr4*$^{+/+}$ and *Tlr4*$^{-/-}$ retinas with severe damage, one-way ANOVA and post hoc Tukey's HSD test). Thus, knocking out *Tlr4* does not prevent RGC death or glaucomatous nerve damage.

Discussion

TLR4 targeting strategies have been studied in multiple models of diseases characterized by an inflammatory microenvironment[29,60]. TLR4 is expressed by a variety of ocular tissues and plays an important role in inflammatory eye diseases[61–63]. A study in mice showed that TLR4 contributes to retinal ischemia/reperfusion injury via NF-kB signaling, increasing the expression of proinflammatory genes[37]. Knockout of *Tlr4* led to a suppressed inflammatory response in the retina. Another study analyzed TLR4 downstream mechanisms and showed that TLR4 signaling mediated by caspase-8 controlled the production of the inflammatory cytokine interleukin 1β (IL-1β)[36]. Inhibition of either TLR4 or caspase-8 blocked the production of IL-1β and attenuated retinal ischemic damage after acute experimental IOP elevation. *Tlr4*$^{-/-}$mice have also been shown to be more resistant to optic nerve crush injury than wild-type mice[34].

Growing evidence suggests that mitochondrial and metabolic disturbances with bioenergetic insufficiency contribute to the demise of RGCs in glaucoma[15,64–67], while an extensive literature implicates neuroinflammatory processes[1,2,4,5]. TLR4 plays a role in multiple metabolic processes, affects mitochondrial function in inflammatory diseases, and has been proposed to have a role in acute glaucoma based on an induced, acute model with very high IOP elevation (ischemia-inducing)[36], but a role in chronic glaucoma is not assessed. Thus, we determined its role in the chronic DBA/2J glaucoma model. Our experiments did not detect any differences in the glaucoma-related phenotype between *Tlr4*$^{-/-}$ and *Tlr4*$^{+/+}$ DBA/2J mice. Both genotypes developed comparable levels of IOP elevation, RGC loss, and optic nerve damage. Our data do not provide evidence for a role of TLR4 in this chronic glaucoma.

The difference between our results and those in more acute models is not surprising as different types and degrees of insult can induce differing damaging mechanisms. For example, in acute glaucoma, there is an overwhelming inflammatory response compared to chronic glaucoma, with slower, more cumulative damage mediated by different pathways (e.g., metabolic failure, oxidative stress, mitochondrial dysfunction, inflammation)[12,15,36,64]. The above-mentioned study in the acute model used an IOP of 110 mmHg[36], and such a degree of IOP elevation does not occur in chronic glaucoma or even in most human patients with acute glaucoma. Additionally, optic nerve crush is meant to model direct axon injury in glaucoma, but there are differences to IOP-induced glaucoma, including transcriptomic differences[68,69]. Another study reported that *Tlr4* mutation protects against TGFβ2- or fibronectin-EDA induced IOP elevation[70]. This finding differs from ours, as TLR4 does not affect IOP in DBA/2J mice. This may reflect a difference between mechanism of IOP elevation following pigmentary insults versus and other forms of IOP elevation.

Our studies indicate that the lack of *Tlr4* does not influence glaucoma progression in DBA/2J glaucoma. Multiple factors can influence disease progression, such as genetic background and environment[71–73], and various factors may influence the effects of TLR4 on glaucoma. TLR4 mediates inflammation in response to infection and endogenous molecules such as saturated fatty acids[74,75]. Thus, the presence of specific infectious or commensal microbes or the nature of diet, including saturated fat content, could possibly impact its role in glaucoma. The immune system often has redundant pathways to ensure robustness. Other TLRs (e.g., TLR2) may compensate for the lack of TLR4[76,77], sustaining the inflammatory response and glaucomatous progression. Additionally, non-TLR pathways, such as the tumor necrosis factor (TNF) signaling pathway, may still drive neuroinflammation and RGC damage independently of TLR4[78,79]. The specific mutation may also play a role[73], with some mutations in the human population possibly being activating. Nevertheless, our studies in a widely used model of chronic glaucoma do not support targeting of TLR4 as a treatment for chronic glaucoma.

Although pathologic neuroinflammation and TLR receptor signaling are implicated in a wide variety of neural diseases and inflammaging[80], our results are not completely surprising. We and others have implicated dysregulated metabolism in glaucoma[12,15,18,20,66,67], while there is growing evidence for reciprocal immuno-metabolic signaling interactions[81,82]. In addition to regulating inflammatory cascades, TLR4 is reported to influence the autonomic control of body temperature, heart rate, and metabolism[83]. Stimulation of TLR4 receptors leads to key phenotypic shifts in astrocytes and microglia, while it reprograms metabolism in both immune cells and glia. This reprogramming results in a shift towards glycolysis[84,85]. Inhibiting this shift has been suggested as a neuroprotective strategy[86]. On the other hand, TLR4 activation also induces AKT, a protein kinase with important roles in modulating both protein synthesis and promoting cell survival[87,88]. This protection involves the kinase TBK1 (in at least dendritic cells), a gene associated with glaucoma[89]. Importantly, TLR9 also has a role in energy metabolism and cell protection as demonstrated in neurons[90], raising the possibility that TLR4 or other related receptors may also promote neuronal protection in specific stress or disease contexts. As further evidence of TLR-mediated protection: (1) deficiency of the TRF1 (important adaptor protein for TLR receptor function) exacerbates amyotrophic lateral sclerosis in mice (proposed astrocyte-related mechanism[91]; (2) while deficiency of MYD88 (another critical adaptor protein) exacerbates excitotoxic cortical neuron injury by kainic acid[92]. The lack of such protective effects in mice completely lacking TLR4 may counteract any beneficial effects of the mutation in lessening neuroinflammation and inflammaging in this glaucoma model. Similarly, lung integrity is compromised in *Tlr4*-mutant mice on at least some strain backgrounds[93], possibly impacting oxygen supply and counterbalancing any beneficial effects. Clearly, further experiments in various models of glaucoma are needed to understand both the damaging and protective roles of TLR receptors/adaptors and reciprocal immunometabolic regulation. It will be important to determine their complex roles in different tissues and cell types at different stages of glaucoma as well as in chronic and acute forms of this disease.

Materials and methods

Ethical approval

All mice were treated in accordance with the Association for Research in Vision and Ophthalmology's statement on the use of animals in ophthalmic research. All animal procedures were performed according to the protocols approved by the Jackson Laboratory and Columbia University's Institutional Animal Care and Use Committee.

ARRIVE guidelines

The study is reported in accordance with the ARRIVE guidelines. Study design: all phenotypes were compared between $Tlr4^{-/-}$ and $Tlr4^{+/+}$ mice. The experimental unit is individual eyes. Sample size is shown in figure legends for each experiment. Given the variability of DBA/2J glaucoma, at least 40 nerves for each genotype were examined when looking at the effect of genotype on neurodegeneration. No data points were excluded. No randomization was used. Both genotypes were housed together and IOP measurements were taken at the same time of the day to minimize potential confounders. Blinding: the researchers were masked for genotypes when conducting experiments, evaluating nerve damage, and counting RGCs. Outcome measures: effect of *Tlr4* genotype on IOP and neurodegeneration. Statistical methods are stated in methods and figure legends.

Mice

B6.B10ScN-$Tlr4^{lps-del}$/JthJ mice (strain #007227) were obtained from The Jackson Laboratory[94,95]. These mice are *Tlr4*-deficient due to lack of exon 3 in the *Tlr4* gene. The *Tlr4* mutation was backcrossed to strain DBA/2J (strain #000671) for >10 generations to produce congenic mice with the *Tlr4*-deficient allele on the DBA/2J background (all experimental mice were ≥N10). For this study, congenic DBA/2J *Tlr4* heterozygote mice were intercrossed to produce *Tlr4* wild-type and homozygous mutant littermates. Both genotypes were housed together and analyzed simultaneously. All mice were housed in a 21 °C environment with a 14-h light and 10-h dark cycle, fed with a 6% fat diet, and acidified (pH 2.8–3.2) drinking water[96]. Both female and male mice were used for analysis.

Clinical slit-lamp examination

Mice underwent regular examinations using a slit lamp bio-microscope at 40X magnification throughout their lifespan, as described in previous studies[47,52,97]. The analysis started at 4 months of age, with subsequent examinations at 6, 8, 10, and 12 months. Phenotypic assessment of iris disease included the evaluation of iris atrophy, pigment dispersion, and transillumination. Mice were examined without anesthesia using standard handling, and all photographs were captured with identical camera settings.

IOP measurement

IOP was assessed using the microneedle method as previously outlined in detail[98,99]. In brief, mice were anesthetized with an intraperitoneal injection of a combination of ketamine (99 mg/kg; Ketlar, Parke-Davis, Paramus, NJ, USA) and xylazine (9 mg/kg; Rompun, Phoenix Pharmaceutical, St Joseph, MO, USA) immediately before the IOP measurement. All IOP measurements for both genotypes were taken at the same time of the day.

Nerve staining and evaluation of damage

The intracranial segments of the optic nerves were fixed in 0.8% paraformaldehyde, 1.22% glutaraldehyde, and 0.08 M Phosphate Buffer pH 7.4 at 4 °C for 12 h, followed by processing and embedding in plastic. The retro-orbital ends of the nerves were cut into 1 µm thick sections and stained with paraphenylenediamine (PPD) as previously described[59,100,101]. PPD stains the myelin sheath of all axons, while specifically darkly staining the axoplasm of damaged axons[101]. At least 3 sections were examined to determine the level of damage for each nerve. The optic nerves were determined to have 3 levels of damage as previously reported and validated against axon counting[9,12]: (1) No or early damage (NOE) - less than 5% of axons were damaged, no gliosis, indistinguishable from no glaucoma controls. These nerves have no damage by conventional criteria but are called no or early as some of them have early molecular changes that precede neurodegeneration. (2) Moderate damage (MOD) - had an average of 30% axonal loss and early gliosis. (3) Severe damage (SEV) - had more than 50% axonal loss and damage with prominent gliosis[4,10,23,100]. All nerves were evaluated by at least two masked investigators. In cases where the two investigators did not agree on the damage level a third investigator (also masked) analyzed the nerve, and the most assigned damage level was used[100].

Retinal wholemounts Nissl staining and cell counting

Retinas from 12-month-old DBA/2J mice were Nissl-stained with cresyl violet as previously reported[57]. In short, eyes were fixed in 4% paraformaldehyde in 0.1 M Phosphate Buffer pH 7.4 overnight at 4 °C and then transferred into 0.4% paraformaldehyde in 0.1 M Phosphate Buffer pH 7.4. Whole retinas were dissected from the eye, processed with 0.3% Triton X-100 and 3% hydrogen peroxide, flat-mounted onto glass slides, and stained for 1 h in 1% cresyl violet in distilled water before being differentiated in 95% alcohol, 100% alcohol, and xylene. Eight 40x brightfield images were obtained (two per quadrant) of peripheral retina, equidistant from the peripheral edge. RGC layer cells were manually counted (endothelial cells excluded) and averaged across all eight images per retina.

Statistical analysis

IOP measurements were compared between $Tlr4^{+/+}$ and $Tlr4^{-/-}$ mice at all ages with a two-way ANOVA. The level of nerve damage between $Tlr4^{+/+}$ and $Tlr4^{-/-}$ mice at each age was compared using Fisher's exact test. RGC numbers were compared with a one-way ANOVA with post hoc Tukey's HSD test.

Data availability

The raw datasets produced during the current study are available from the corresponding author upon reasonable request.

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Author contributions

S.W.M.J. conceived the project; C.Z., J.M.H., and S.W.M.J. designed the research; C.Z., H.L., Q.W., M.S., J.M.H., and S.W.M.J. performed research and analyzed data; C.M. managed mouse colony; C.Z., M.S., and S.W.M.J. wrote the manuscript. All authors read and edited the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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